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Atty. Docket No. CRP-008DV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): Huston, James S. et al. EXAMINER: J. Ulm

SERIAL NO.: 07/661,070 GROUP NO.: 185

FILED: February 26, 1991

TITLE: PRODUCT AND PROCESS FOR THE PRODUCTION, ISOLATION AND PURIFICATION OF RECOMBINANT POLYPEPTIDES

which is a DIVISIONAL OF: 07/462,297, FILED: 12/28/89
which is a CONTINUATION OF:07/028,484, FILED: 3/20/87

Honorable Commissioner of Patents
& Trademarks
Washington, D.C. 20231

DECLARATION OF DAVID C. RUEGER, MARC F. CHARETTE, CHARLES M. COHEN, ROBERTO CREA, JAMES S. HUSTON, PETER C. KECK, HERMANN OPPERMANN, AND RICHARD J. RIDGE UNDER 35 U.S.C. RULE 1.131

We declare:

1. We invented the subject matter claimed in the above-referenced application, which claims priority as a divisional application from USSN 07/462,297, filed 12/28/89, which in turn is a continuation of 07/028,484 filed 3/20/87. We, Drs. Rueger, Charette, Cohen, Huston, Keck, and Oppermann, are employees of the named assignee, Creative BioMolecules, Inc. I, Dr. Ridge, am a former employee of Creative BioMolecules, Inc. I, Dr. Crea, am a former employee and a current member of the Board of Directors of Creative BioMolecules, Inc.

2. Prior to March 28, 1986, we reduced to practice in the United States the construction and expression of recombinant DNA encoding a fusion polypeptide containing a leader/hinge region, a cleavage site, and a target polypeptide, and the subsequent cleavage of that polypeptide at the cleavage site by a cleavage site-specific protease. The following experiments, performed by Zita Babickas under the direction of David C. Rueger, demonstrate that a fusion polypeptide having the structure described in the above-captioned patent application is cleaved at the cleavage site nearest the hinge region. These experiments are described in the above-captioned patent application (see Example III, in particular) and in the accompanying Exhibits A and B. The results are shown in Exhibits C-E. These experiments involve fusion polypeptides in which the target protein is calcitonin.

3. Polypeptide 1, described on pages 39-41 of the above-captioned patent application, includes a TRP-LE leader sequence connected at its carboxyl end to a Phe-Pro-Gly hinge, followed by a glutamic acid residue and human calcitonin. As stated in the specification on page 40, lines 1-7, it was desired that the tripeptide hinge would promote cleavage at the Glu residue by providing a flexible residue (glycine) and uncharged "spacer" residues next to the Glu residue. The large leader was used to promote high expression levels. The sequence of polypeptide 1 is provided in the specification on pages 40-41.

4. Polypeptide 2, described on pages 40-41 of the above-captioned patent application, is a smaller polypeptide in which the TRP-LE leader is truncated, a dipeptide Asp-Leu hinge, a Glu cleavage site, and salmon calcitonin analog as a target polypeptide. The salmon calcitonin analog was used because it contained an Asp at position 15 instead of the cleavable native Glu. The sequence of polypeptide 2 is set forth on page 41 of the specification. As stated in the specification on page 41, polypeptide 2 was designed with the goal of obtaining a fusion polypeptide which was cleavable into only 2 fragments, the hook/hinge fragment and calcitonin. Also, the hinge of polypeptide 2 was designed to facilitate cleavage with V-8 protease such that cleavage approached 100% completion.

5. Exhibits A and B, attached hereto, demonstrate the experiments in which polypeptides 1 and 2, respectively, were cleaved with V-8 protease. These experiments were performed and the pages submitted as Exhibits A-E are dated prior to March 28, 1986. Exhibits C, D, and E show the results of these cleavage experiments. Exhibit C shows cleavage of polypeptide 1, in which V-8 protease cleaved the fusion protein into multiple fragments. Exhibit D shows cleavage of polypeptide 2, in which V-8 protease cleaved the fusion protein into the two expected fragments, identified as "hook" (containing the leader and hinge regions) and "SC" (containing the salmon calcitonin analog). Exhibit E

shows the purified salmon calcitonin analog collected from the "SC" peak shown in Exhibit D.

6. All statements made herein of our own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1011 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

9/29/92
Date
David C. Rueger

9/28/92
Date
Marc F. Charette

9/25/92
Date
Charles M. Cohen

9-25-92
Date
Roberto Crea

9-29-92
Date
James S. Huston

9-28-92
Date
Peter C. Keck

9-25-92
Date
Hermann Oppermann

8-20-92
Date
Richard J. Ridge

189kam2054/6.AH6

PROJECT Calcitonin 133 polypeptide 1

Continued From Page

Staph cleavages (bulk) of LE-Glu-HC in 4M+6M urea w+w/o DTT

2 ml of C130-pool were dialized overnight vs 6M urea Ac2O E, pH 5
Concentration assumed @ .7 mg/ml as before

code	µl prot	µl buff	mg prot	ratio	µl enzyme
133-A	500	-	.355	1:30	6.7
-B*	330	170	.234	1:50	4.7

another shot of 1:50 enzyme added after 2hr. (1:30 pm) (C133-A,B-2hr+0

Gel samples taken @ 2hr, 2hr+5hr, 2hr+28hr.

12/84 another shot of 1:50 enzyme added after 21 hrs (1:30 pm 9/12) (C133-A,B-2hr+17
4µl of staph added to A @ 5:30pm (C133-A-2hr+24
+6+8hr.

code	µl prot	µl buff	µl DMA/DTT	mg prot	ratio	µl enzyme
133-C*	200	100	-	.142	1:50	2.84
-D*	200	100	10	.142	1:50	2.84

protein incubated w/ DTT for 2 hrs before enzyme addition - 2nd DTT final conc
extra shot of 1:50 added after 3hrs.
C received 2nd extra shot after 3 hrs. (C133-C-2hr-3hr+x) =
D " " " " 18 hrs (C133-D-2hr+18hr+x)

12/13/84

code	µl prot	µl buff	mg prot	ratio	µl enzyme
133-F*	1.0	.5	.7	1:50	13.4

extra shots after 2hr + 18 hrs (C133-F-2hr+18hr+x)
pH was ~7.1 after 18hr - brought up to pH 7.9 before addition of enzyme

All 4M urea samples were turbid after ~1hr

Yield: 200µl collection (94.6µg injected x .14 (14%) = 13.2µg calc corrected)
peak weight = 7.5% of total = 7.1µg = 53.8%
" OD = .37 ÷ .035 µg = 10.6µg = 80.3%

Continued on Page

Read and Understood By

Rita A. Babickas C.H.

PROJECT Calcitonin 180 NOTEBOOK NO. polypeptide 2

Continued From Page

Bulk Staph cleavage of reduced Synthetic SC-deep

2 ml of conc. C178-pool made 20mM DTT + Left @ RT for 2 hrs
Dialyzed overnight vs 1L Ce Mures 20M NHy Acet E, pH 7.8 (1.3 mg/ml)

4/7/85

(A) 100 µl of protein (assume mg/ml) + 50 µl 20mM NHy Acet E, pH 7.8
+ 3 µl staph (Pierce .64 mg/ml) 1:50 excess @ 9:50 (1:70 really)
collected @ 4.5' + 1¼ hrs

(B) 200 µl of protein (.26 mg) + 100 µl 20mM NHy Acet E, pH 7.8
+ 2.7 µl staph (Pierce .64 mg/ml) 1:150 excess @ 12:10
collected @ 30', 1 hr + 1½ hrs

(C) 200 µl of protein (.26 mg) + 100 µl 20mM NHy Acet E, pH 7.8
+ 2.7 µl staph (Pierce .64 mg/ml) : 1:150 excess @ 2:00
collected @ 30', 1 hr + 1½ hrs.

(D) 200 µl of protein + 100 µl 20mM NHy Acet E, pH 7.8
+ 2 µl staph (Pierce .64 mg/ml) 1:200 excess @ 4:35
left 4°C after ~2 hrs

Yield: ~90 µg of cSC / collection on LC (100 µl of each digest)
∴ 90 mg / 1 cell conc C178-pool
∴ 9.5 mg cSC / 10 gr cells.

~ 1 mg cSC / g. cell

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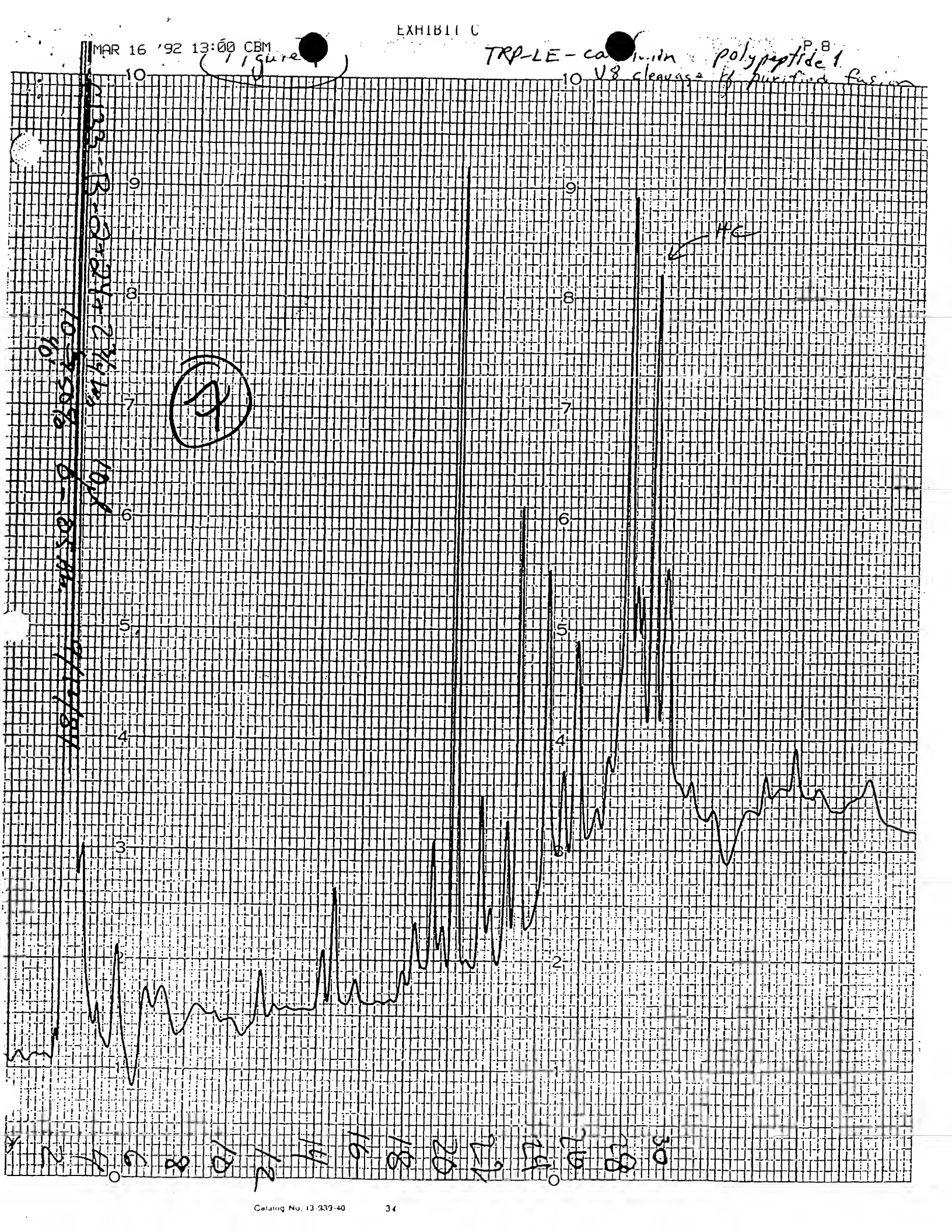
Read and Understood By

Peter A. Peterkofsky C.H.

Figure 1

TRP-LE-ca... polypeptide 1
V8 cleavage of purified fus...



TRIPLE + calcitonin polypeptide 2 V8 cleavage of purified fusion

Figure 2

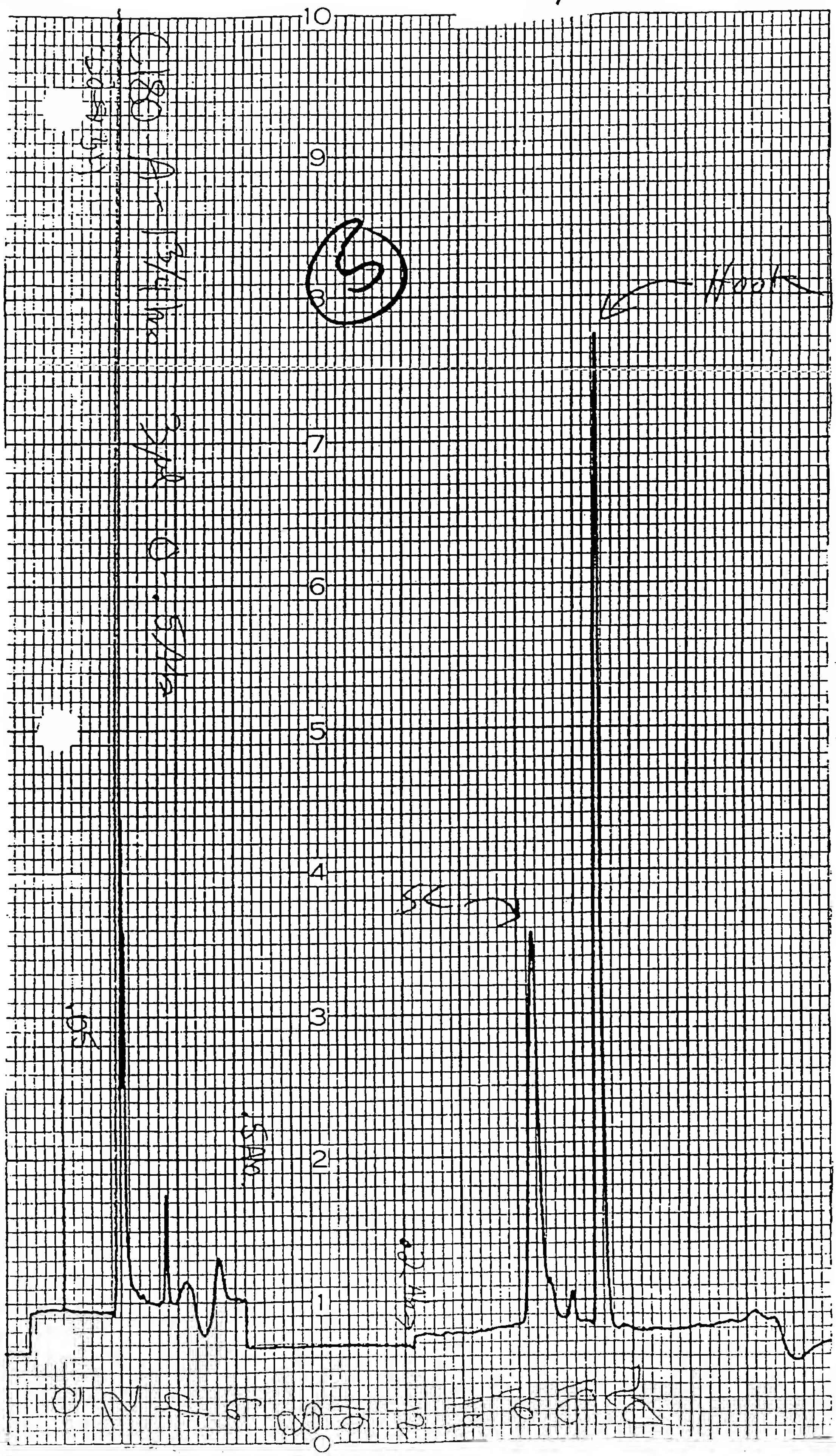
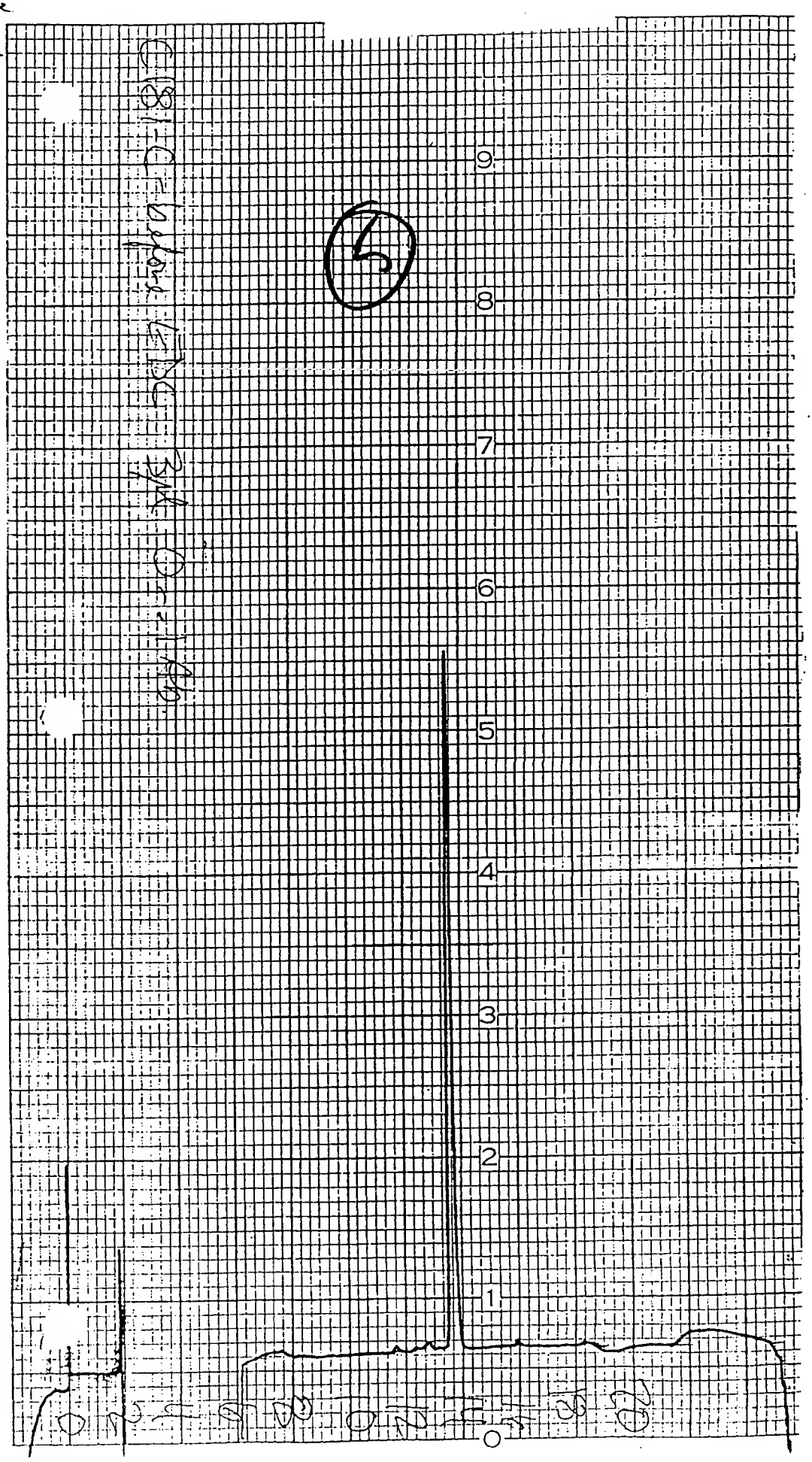


EXHIBIT E

Figure 3

polypeptide 2 vs cleavage purified calcitonin



The identity of the calcitonin analog released from polypeptide 1 after V-8 protease cleavage has been confirmed by sequence analysis to be Phe-Met-Arg-calcitonin. The chromatogram from HPLC analysis of such a digest on a C-18 column developed with a acetonitrile/trifluoroacetic acid (0.1% TFA) gradient is complex since the fusion protein has a molecular weight of about 124,000 daltons (leader approximately 120,000 daltons, hinge calcitonin, approximately 3500 daltons) and contains numerous Glu residues. The calcitonin analog was shown by Western blot analysis to correspond to a specific fraction in the HPLC separation. The fraction was then rechromatographed on a C-8 column yielding pure Phe-Met-Arg-calcitonin.

Isolated Phe-Met-Arg-calcitonin can be cleaved to yield free calcitonin using endo-Arg C protease. The calcitonin can then be isolated by C-18 column chromatography. Its identity has been confirmed by sequence analysis and tryptic peptide mapping.

Example III

A series of fusion polypeptides comprising calcitonin and calcitonin analogs linked to hook polypeptides of various designs were expressed in E. coli. Each fusion protein had designed hook and hinge regions. Each hook contained a glutamic acid residue immediately preceding the N-terminal cysteine residue of calcitonin. The calcitonin was designed to be released from the fusion by cleavage at the Glu residue by V-8 protease.

Polypeptide I had a TRP-LE sequence (188 residues) as a leader, connected at its carboxyl end to a hinge (Phe-Pro-Gly) region, followed by the glutamic acid residue and human calcitonin. It was

hypothesized that this hinge tripeptide would promote cleavage at the Glu residue by providing a flexible residue (proline) and an uncharged "spacer" residue next to the Glu residue. The large leader (27,000 daltons) was used to promote high expression levels. Small leader sequences such as a 17-residue truncated TRP-LE resulted in no expression.

Polypeptide 2 had a modified truncated TRP-LE sequence comprising in sequence a truncated TRP-LE (15 residues) followed by 43 residues derived from the TRP leader sequence (which had been modified by conventional techniques to change the native 3 Met residues to Val, the native 4 Glu residues to Asp, and the single Cys residue to Ser), Asp-Leu as the hinge, the Glu cleavage site, and a salmon calcitonin analog. The salmon calcitonin analog had an Asp at position 15 instead of the native Glu. The internal Glu was discovered to be easily cleaved by V-8 protease. Construction of a calcitonin analog sought to obviate this difficulty. A Gln 15 analog was initially constructed but the fusion expression level was very low. An Asp 15 analog constructed subsequently demonstrated high expression levels.

The structures of polypeptide 1 and 2 are set forth below, the underlined residues indicating desired cleavage sites, the overlined residues indicating the hinge, HC indicating human calcitonin, and SC the salmon calcitonin analog.

Polypeptide 1

MET LYS ALA ILE PHE VAL LEU LYS GLY SER LEU ASP ARG
ASP LEU ASP SER ARG ILE GLU LEU GLU MET ARG THR ASP
HIS LYS GLU LEU SER GLU HIS LEU MET LEU VAL ASP LEU
ALA ARG ASN ASP LEU ALA ARG ILE CYS THR PRO GLY SER
ARG TYR VAL ALA ASP LEU THR LYS VAL ASP ARG TYR SER

TYR VAL MET HIS LEU VAL SER ARG VAL VAL GLY GLU LEU
ARG HIS ASP LEU ASP ALA LEU HIS ALA TYR ARG ALA CYS
MET ASN MET GLY THR LEU SER GLY ALA PRO LYS VAL ARG
ALA MET GLN LEU ILE ALA GLU ALA GLU GLY ARG ARG ARG
GLY SER TYR GLY GLY ALA VAL GLY TYR PHE THR ALA HIS
GLY ASP LEU ASP THR CYS ILE VAL ILE ARG SER ALA LEU
VAL GLU ASN GLY ILE ALA THR VAL GLN ALA GLY ALA GLY
VAL VAL LEU ASP SER VAL PRO GLN SER GLU ALA ASP GLU
THR ARG ASN LYS ALA ARG ALA VAL LEU ARG ALA ILE ALA

THR ALA HIS HIS ALA GLN GLU PHE PRO GLY GLU HC

Polypeptide 2

MET LYS ALA ILE PHE VAL LEU LYS GLY SER LEU
ASP ARG ASP LEU ASP SER ARG LEU ASP LEU ASP
VAL ARG THR ASP HIS LYS ASP LEU SER ASP HIS
LEU VAL LEU VAL ASP LEU ALA ARG ASN ASP LEU
ALA ARG ILE VAL THR PRO GLY SER ARG TYR VAL

ALA ASP LEU GLU SC

Smaller hook sequences were also designed, but resulted in little or no expression of the fusion polypeptides. Polypeptide I demonstrated good expression levels (15-20%), but because of the presence of multiple Glu and Cys residues in the leader sequence, the post-digestion mixture was complex, containing many fragments, including disulfide linked combinations of fragments. Thus, it was desirable to design a fusion polypeptide that was as small as possible to achieve good expression and could be cleaved into only 2 fragments (the hook/hinge fragment and calcitonin) by V-8 protease. Polypeptide 2 was designed with that goal in mind.